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Application of Fluorescent In Situ Hybridization With X and Y Chromosome Specific Probes to Buccal Smear Analysis

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Conventional X- and Y-chromatin and fluorescent in situ hybridization (FISH) analysis based on X- and Y-chromosome specific probes were conducted from buccal smear, on 15 normal males, 15 normal females, and 9 cases suspected of sex chromosome anomalies. The proportion of X- and Y-chromatin in normal females and males was 12% ± 3% and 51.5% ± 4.9%, respectively, by the conventional X- and Y-chromatin procedure. The CEP-X/Y analysis by FISH for the same specimens provided a proportion of 98.8% ± 0.7% cells with XX signals in the normal females and 99.8% ± 0.4% cells with XY signals in the normal males. The FISH method was superior to the conventional procedure in nine cases suspected of sex chromosome anomalies, including one case of mosaicism. The results of CEP-X/Y will sometimes be false; it will not detect structural anomalies of sex chromosomes, and it is not intended to detect low level mosaicism. However, the test is useful for rapid screening of sex chromosome aneuploidy at a fraction of the cost for chromosome analysis. The FISH test is also appropriate to detect tissue specific sex chromosome mosaicism, especially if it is relatively high. This FISH test is best used as an adjunct to chromosome analysis whenever possible. © 1996 Wiley-Liss, Inc.

KEY WORDS: buccal smear, FISH, sex chromosome aneusomy

INTRODUCTION

Conventional X- and Y-chromatin analysis of buccal cells have been in use for determination of sex chromosome aneuploidy since the sixties. Because X chromatin can be identified in about 12% to 20% of buccal cells in normal females, this method is not sensitive for detection of X chromosome mosaicism and often difficult in establishing cases of aneusomy. Unfortunately, this kind of mosaicism is relatively common, especially among Ullrich-Turner syndrome patients [Held et al., 1992]. The Y-chromatin test is more sensitive than the X-chromatin, but it identifies Y chromosome in only about 50% of buccal cells in normal males. Furthermore, the Y-chromatin test relies on the presence of heterochromatin (band Yq12), but since the size of this band varies extensively among males it can occasionally lead to erroneous results.

The introduction of fluorescent in situ hybridization (FISH) and chromosome specific DNA probes have greatly enhanced the sensitivity of detection of X and Y chromosomes from interphase cells of a variety of tissues ranging from bone marrow [Dewald et al., 1993], buccal cells [Harris et al., 1994], sperm [Lu et al., 1994], amniocytes [Gersen et al., 1995; Divane et al., 1994], and chorionic villi [Rao et al., 1993]. We have investigated the efficacy of FISH using probes specific for X and Y chromosomes for a study of buccal cells from a series of normal individuals and those suspected of having sex chromosome abnormalities. These probes were directly labeled with different color fluorophores so that the X and Y chromosome signals could be observed simultaneously. This FISH technique does provide a vastly improved test to the conventional method of buccal smear analysis for numeric anomalies, but has some of the same limitations of the conventional method for structural anomalies of the sex chromosomes.

MATERIALS AND METHODS

Buccal mucosa cells were collected from 30 normal individuals (15 females and 15 males) and 9 patients suspected of sex chromosome anomalies (Fig. 1). A sterile metal spatula or a cytopak brush was used to scrape

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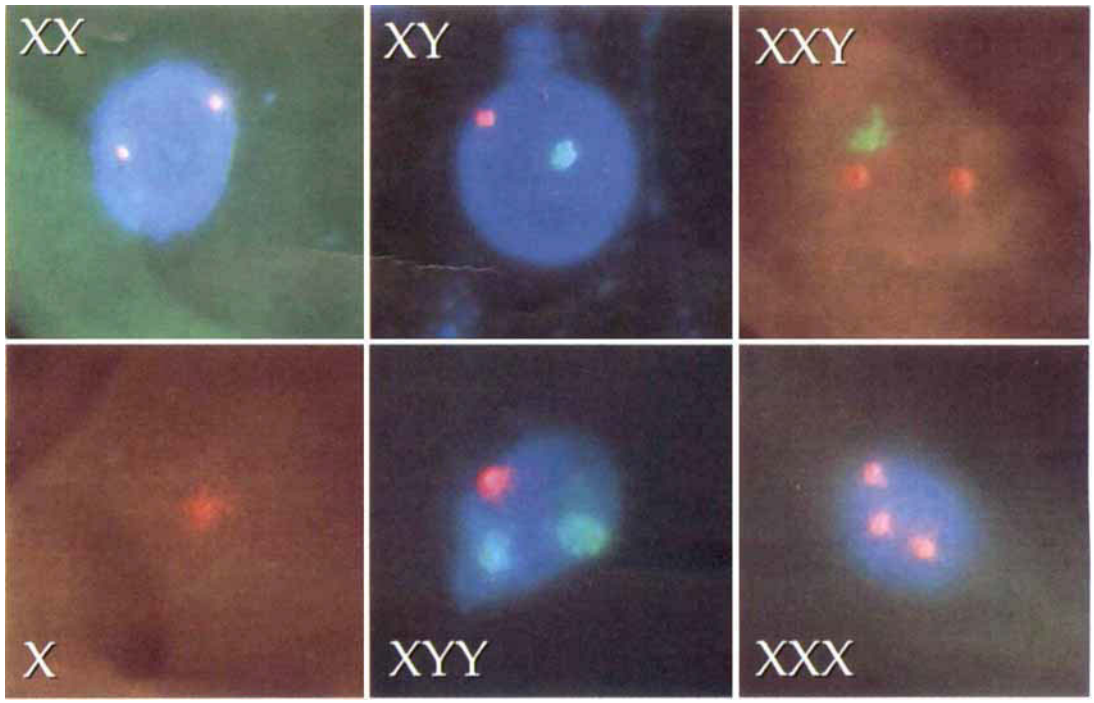

Fig. 1. Buccal smear cells from normal female, normal male and sex chromosome aneusomies by fluorescent in situ hybridization. The directly labelled alpha satellite for X centromere is in Spectrum Orange and most of the directly labelled Yq-arm is in SpectrumGreen. The background is either DAPI (blue) or propidium iodide (orange). The massive cytoplasm of the buccal cells have an unusual hue from these counterstains.

the right and left cheek of each individual. The first scraping from each cheek was discarded to minimize bacterial contamination. The buccal cells were spread across a clean glass slide, fixed for 1 hour in methanol:glacial acetic acid fix (3:1), air dried, and stored at −20°C. Alternatively, the cytopak brush was stored in a screw cap tube for transportation to the laboratory prior to the slide preparation.

Conventional X- and Y-chromatin analysis [Mittwoch, 1974; Pearson et al., 1970] and FISH studies were performed on each individual. Two microscopists (D.G.K., W.A.W.) were unaware of the specimen identification during the analysis. For conventional X- or Y-chromatin studies, two slides (one from each cheek) of each patient were stained for 5 minutes with 1% cresyl violet acetate (in 50% ethanol), rinsed three times in 95% ethanol, rinsed twice in xylol, placed in fresh xylol for 2 minutes, and then air-dried. For each person, the number of X-chromatin bodies was scored from 200 buccal nuclei at ×1,250 by transmitted light microscopy. An X-chromatin was defined as a dark-staining body that was touching the nuclear membrane. We scored only intact nuclei that were not folded or were not excessively granular.

For Y-chromatin analyses, two slides (one from each cheek) for each sample were stained in 50 μg/mL quinacrine mustard, rinsed in deionized water, and stored in Sorensen phosphate buffer (pH 6.8) for 1 minute. The excess buffer was removed from the slide surface with a paper towel, two drops of *n*-propyl gallate (2.5% in glycerol) were placed on the slides, and a coverslip was applied. For each person, the number of Y-chromatin bodies was scored from 200 nuclei at ×1,250 with an incident-light fluorescence system.

Buccal smear slides used for FISH were warmed to room temperature before use. The slides were pretreated with 0.01% collagenase-H (Sigma Chemical, St. Louis, MO) in Krebs-Ringer's solution for 1 hour at 30°C, washed three times for 2 minutes each in 2× SSC (300 mM/L sodium chloride, 30 mM/L sodium citrate), fixed for 10 minutes in 1% formaldehyde/50 mM/L magnesium chloride/1× PBS (130 mM/L sodium chloride, 13 mM/L sodium phosphate dibasic, 3 mM/L sodium phosphate monobasic), and then washed for 6 minutes in 1× PBS/50 mM/L magnesium chloride.

Directly labeled probes specific for X and Y chromosomes (CEP-X/Y) were purchased from Vysis (formerly Imagenetics), Naperville, IL. The X-chromosome probe was centromere-specific alpha satellite DNA, labeled with SpectrumOrange. The Y-chromosome probe was a collection of DNA segments labeled with SpectrumGreen, which hybridized to most of Yq11.2 and all of Yq12. A hybridization solution consisting of 2 μL of CEP-X/Y, 7 μL of Hybridization Mix 3 (Vysis), and 1 μL of distilled water was used for each FISH assay. Probe hybridization was accomplished by placing the hybridization solution on each slide, and placing a 22 by 22 mm coverslip over it which was sealed with rubber cement. Chromosome and probe DNA were denatured simultaneously at 80°C for 2 minutes and hybridized for 1 hour at 50°C. Coverslip was removed and each

slide was then washed three times for 10 minutes each with 50% formamide/2× SSC at 45°C, once with 2× SSC at 45°C for 10 minutes, and once with 2× SSC/0.1% NP-40 (Nonidet P-40) at 45°C for 10 minutes. Slides were then air-dried and counterstained with 7.5 μL of 4′,6′-diamino-2-phenylindole dihydrochloride (DAPI) (Vysis).

For each specimen, we scored 200 nuclei with one or more FISH signals. Overlapping or broken nuclei were not used in the analysis. The cells were viewed with an incident-light fluorescent microscope at ×1,000 with either a dual- or triple-pass filter. One dual-pass filter was for fluoroisothiocyanate/propidium iodide (Chromatechnology Corporation, Brattleboro, VT) to view the SpectrumGreen and SpectrumOrange signals. A second dual-pass filter set, IO2/IO4 (Vysis Filters, Naperville, IL), was used to view the DAPI counterstain and the SpectrumOrange signals simultaneously. A triple-pass filter, DAPI/IO2/IO4 (Vysis Filters), was used to view all three fluorescent signals simultaneously. With these combinations of filters, an X-chromosome centromere exhibited an orange signal, a Y q-arm exhibited a green signal, and the background stained blue (Fig. 1). We used either Ektachrome 400 film to photograph representative cells or unenhanced digital images from image analysis systems.

RESULTS

The normal ranges for conventional X- and Y-chromatin analysis and FISH studies were established from 30 individuals. For the 15 normal females, the mean percentage of cells with X chromatin was 12% ± 3% and the range was 7% to 17% (Table I). In these same specimens, the mean percentage of cells with two X signals by CEP-X/Y analysis was 98.8% ± 0.7% and the range was 97.5% to 100%. There were no cells with just two X signals in the normal males.

For the 15 normal males, the mean percentage of cells with Y chromatin by the conventional procedure was 51.5% ± 4.9% and the range was 42.5% to 60% (Table II). With CEP-X/Y analysis for these same specimens, the mean percentage of cells with an XY signal pattern was 99.8% ± 0.4% and the range was 99% to 100%. In male 4, 0.5% of the cells had a single X signal. In males 1 and 2, an XXY signal pattern was seen in 1% and 0.5% of the cells, respectively. There were no cells with just two X signals in the normal males.

A normal range using the upper bound of a one-sided 95% confidence interval was calculated for the proportion of "aberrant" interphase cells based on a binomial distribution with n = 200. For the purpose of this study, "aberrant" refers to cells that were other than XX in females or other than XY in males. Given that 200 cells were analyzed and the proportion of 3, 4, or 5 cells other than XX in normal females or XY in normal males, the corresponding upper 95% confidence bounds are 3.8%, 4.5%, and 5.2% respectively. Assuming no more than five aberrant cells in normal females or males, the upper limit of the normal range was set at 5.2% for this study.

Buccal smears from nine patients suspected of having sex chromosome abnormalities were also investigated (Table III). Cases 2, 3, 4, 5, and 7 had a karyotype of 46,XY. FISH analysis of buccal smear cells indicated that 98.2 to 100% of the cells had an XY signal pattern,

TABLE I. Analysis of X and Y Chromosome by the Conventional Method and by CEP-X/Y From Buccal Smear Interphase Cells From 15 Normal Females

	CEP-X/Y (%)[a]		X and Y chromatin[b]	
Case	XX	X	% cells with one X-chromatin	% cells with one Y-chromatin
1	97.5	1.5	12	0
2	98.0	1.5	13	0
3	98.0	2.0	16	0
4	98.0	2.0	11	0
5	98.5	1.0	14	0
6	98.5	1.5	12	0
7	99.0	1.0	13	0
8	99.0	1.0	15	0
9	99.0	1.0	17	0
10	99.0	1.0	16	0
11	99.0	1.0	12	0
12	99.5	0.5	7	0
13	99.5	0.5	8	0
14	99.5	0.5	9	0
15	100.0	0.0	15	0
Mean	98.8	1.1	12	0
± SD	0.7	0.6	3	0
Min	97.5	0.0	7	0
Max	100.0	2.0	17	0

[a]Analysis of 200 cells with the CEP-X/Y probe mixture (total is not always 100% to account for >2 X signals).
[b]Analysis of 200 cells for X chromatin and 200 cells for Y chromatin.

TABLE II. Analysis of X and Y Chromosome by the Conventional Method and by CEP-X/Y From Buccal Smear Interphase Cells From 15 Normal Males

	CEP-X/Y (%)[a]		X and Y chromatin[b]	
Case	XY	XXY	% cells with one X-chromatin	% cells with one Y-chromatin
1	99.0	1.0	0	48.0
2	99.0	0.5	0	56.0
3	99.5	0.0	0	43.0
4	99.5	0.0	0	55.0
5	100.0	0.0	0	46.0
6	100.0	0.0	0	52.0
7	100.0	0.0	0	49.0
8	100.0	0.0	0	60.0
9	100.0	0.0	0	50.0
10	100.0	0.0	0	54.0
11	100.0	0.0	0	54.0
12	100.0	0.0	0	58.0
13	100.0	0.0	0	56.0
14	100.0	0.0	0	47.0
15	100.0	0.0	0	49.0
Mean	99.8	0.1	0	51.5
± SD	0.4	0.3	0	4.9
Min	99.0	0.0	0	42.5
Max	100.0	1.0	0	60.0

[a]Analysis of 200 cells with the CEP-X/Y probe mixture (total is not always 100% to account for single X or XXYY signals).
[b]Analysis of 200 cells for X chromatin and 200 cells for Y chromatin.

TABLE III. Buccal Smear Analysis of X and Y Chromosome by the Conventional Method and by CEP-X/Y in Patients Suspected of Sex Chromosome Anomalies*

Case	Reason for referral	Karyotype[a]	Age, years	Sex	Cep-X/Y[b] (%)							X and Y chromatin[c]	
					X	XX	XY	XXY	XXX	XYY	XXYY	% cells with one X chromatin	% cells with one Y chromatin
1	r/o Klinefelter syndrome	Not done	29	M	0	0	3.6	95.4	0	0	1.0	17.5	52.5
2	XY female	46,XY	5	F	0.6	0	99.0	0	0	0	0	0	57.5
3	XY female	46,XY	14	F	0.4	0	99.4	0	0	0	0.2	0	52.5
4	XY female	46,XY	6	F	0	0	100.0	0	0	0	0	0	54.5
5	r/o Klinefelter syndrome	46,XY	67	M	0.4	0	98.2	1.0	0	0	0.4	0	56.0
6	Premature ovarian failure	45,X (76%) 46,XX (2%) 46,XXX (22%)	38	F	12.1	5.8	0	0	82.1	0	0	38.0[d]	0
7	Ambiguous genitalia	46,XY	1 day	M	0	0	99.5	0	0	0.5	0	0	4.0
8	Azoospermia	46,X	31	M	0	100.0	0	0	0	0	0	11.5	0
9	Short Stature and unspecified dysmorphic features	45,X (43%) 46,X,i(Xq) (57%)	53	F	1.0	99.0	0	0	0	0	0	10.5	0

*r/o, rule out.
[a]All karyotypes from blood specimens.
[b]Analysis of 200 cells with the CEP-X/Y probe mixture.
[c]Analysis of 200 cells for X chromatin and 200 cells for Y chromatin.
[d]Includes cells with one and two X-chromatin bodies.

whereas the cells with one Y-chromatin by conventional buccal smear analysis ranged from 4.0 to 57.5%. Case 8 had a karyotype of 46,XX by cytogenetic analysis of 30 cells from a blood specimen. Analysis of buccal smear cells by conventional techniques showed 11.5% of cells with one X-chromatin, but FISH analysis of this same specimen indicated that 100% of the cells analyzed had a XX signal pattern.

Case 6 was a complex mosaic based on cytogenetic study of peripheral blood, skin, and ovarian tissue. In the blood, 76% of the cells were 45,X, 2% were 46,XX, and 22% were 47,XXX. Chromosome studies of fibroblast cultures from skin and ovary biopsy specimens indicated the presence of only 45,X (100%) in skin. However, 45,X and 47,XXX (50% and 50%) were present in ovarian cells. The CEP-X/Y results indicated that 12.1% of the buccal cells had a single X chromosome, 5.8% had two X chromosomes, and 82.1% had three X chromosomes. Conventional buccal smear analysis indicated that 25% of cells had one X-chromatin and 13% had two X-chromatins.

Case 1 was referred to rule out Klinefelter syndrome. Although no specimen was provided for chromosome studies, CEP-X/Y analysis of buccal smear cells indicated that 95.4% of the cells had a sex-chromosome complement of XXY. Conventional buccal smear analysis showed that 17.5% of cells had X-chromatin and 52.5% of cells had Y-chromatin.

Case 9 was referred for short stature and unspecified minor anomalies. The karyotype was mos45,X(43%)/46,X,i(Xq)(57%). The buccal smear analysis by FISH detected 99% XX cells, and by conventional analysis the proportion of X chromatin was 10.5%. Apparently, mosaicism of 45,X cell line was not present in the buccal tissue.

The XYY figure (Fig. 1) is from an occasional cell seen in males.

DISCUSSION

This study indicates that the use of FISH with directly labeled probes specific for X and Y chromosomes (CEP-X/Y) is more reliable than conventional X- and Y-chromatin studies for detection of aneuploidy. The X and Y signals were large and readily distinguishable from one another (Fig. 1), and thus the scoring procedure was easy and accurate. The proportion of cells with scorable sex chromosome complement was vastly higher with CEP-X/Y than conventional X- and Y-chromatin studies. This observation was evident in both the 30 normal controls and the 9 patients suspected of having sex chromosome abnormalities. The conventional method may be false negative if Yq12 is very small or missing, but FISH will not have this problem since the probe hybridizes to Yq12 and about half of Yq11.2, although the hybridization signal will be much smaller.

In our series of 30 normal controls, the largest number of aberrant cells was 5 in any one female and 2 in any one male. Thus, we could have used an upper limit cutoff of 5.2% for normal range of aberrant cells. However, the percentage of cells with "sporadic" sex chromosome aneuploidy in normal individuals (especially adults) may be higher [Horsman et al., 1987; United Kingdom Cancer Group, 1992] Thus, mosaicism of less than 10% needs to be interpreted with a great deal of caution.

Harris et al. [1994] used buccal smear analysis by FISH in four normal individuals and confirmation of a patient with trisomy 18 and a patient with trisomy 21. They report a probe efficiency of 71% ± 6.8%. In this study, the probe efficiencies was 99.3% ± 0.6% for CEP-X/Y. The difference may be due to a smaller sample size and use of indirect labeled cosmid probes by Harris et al.

Mosaicism is a common condition among patients with a chromosome abnormality involving the sex chromosomes. Our studies of the normal controls indicated that FISH with chromosome-specific probes should be sufficiently accurate to detect cell lines constituting >6% of the total cell population. In case 6, only 2% of cells from a blood specimen had a karyotype of 46,XX, yet 5.8% of cells had two X signals by FISH analysis of buccal cells. This finding suggests that CEP-X/Y analysis may be sensitive to detect clinically significant mosaicism, which is often not possible by conventional technique for buccal smear analysis.

The FISH technique seems to work even when the buccal smear preparation is of suboptimal quality. The specimen from case 7 was relatively poor. This condition probably affected the results of the conventional X- and Y-chromatin studies, as just 4% of the cells were Y-chromatin-positive. This finding was well below the normal limit for our laboratory. However, CEP-X/Y analysis indicated a sex chromosome complement of XY in 99.5% of buccal cells. This observation was confirmed by the cytogenetic analysis of 30 metaphases from a blood specimen.

Buccal cells are derived from ectoderm. Thus, if the results of standard chromosome studies on blood or fibroblast cultures suggest mosaicism but are equivocal, buccal cells can be an important alternative tissue to study. The extent of mosaicism is known to vary significantly among different tissues in some patients. This variability may explain why the percentage of cells from the different cell lines was different in the blood, ovary, skin, and buccal smear specimens from case 6. Because this information can have clinical importance, FISH can be a useful method to study tissue-specific mosaicism.

One of the major sources of false positive result for the Y signal using CEP-X/Y could be the occurrence of Y long arm heterochromatin translocation to the p-arm of acrocentric chromosomes especially chromosomes 15 and 22. Though these are normal polymorphic variants, their frequency of occurrence could be as high as 1:2,000 [Alitalo et al., 1988]. In addition, the use of CEP-X/Y for interphase cytogenetic analysis will not make a distinction between a normal X and i(Xq) (case 9 of this study), r(X) or other structurally altered X chromosomes. Thus, a significant proportion of Ullrich-Turner syndrome patients who have structurally abnormal X or a segment of the Y will not be properly interpreted without a metaphase based chromosome analysis. We have reviewed 169 consecutive blood specimens referred to our laboratory to either rule out "Klinefelter syndrome" or "Turner syndrome." Chromo-

some analysis for these 169 specimens indicated that >75% (128/169) had a normal karyotype consistent with the sex of the patient. Buccal smear analysis using CEP-X/Y would have correctly identified >97% (165/169) of the sex chromosome complement. Two patients had autosomal anomalies that would have required standard chromosome analysis for detection. Two others had i(Xq) that the interphase FISH strategy would miss. Thus, the FISH strategy for buccal interphase cells using CEP-X/Y works well as a rapid screening option for aneuploidy involving the sex chromosomes. The FISH strategy will also be clearly useful when karyotyping is not possible. The cost of this FISH test is about a third of the standard chromosome analysis.

The buccal cells can also serve as alternative noninvasive tissue source to rule out sex chromosome mosaicism, especially if it is above 10%. However, due to the limitations inherent in interphase cytogenetics and the DNA probes in use, we recommend a follow-up of complete chromosome analysis whenever possible.

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